Exploring deep microbial life in coal-bearing sediment down to ~2.5 km below the ocean floor

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Abstract: Microbial populations inhabit deeply buried marine sediments, but the extent of this vast ecosystem is poorly constrained. Here we provide evidence for the existence of microbial communities in sediment associated with lignite coalbeds at ~1.9 to 2.5 km below the seafloor in the Pacific Ocean off Japan. Microbial methanogenesis is indicated by isotopic data of methane and carbon dioxide, methanogenic biomarkers, cultivation and gas composition results. Rigorous protocols aimed at minimizing and correcting for sample contamination resulted in indigenous biomass estimates as low as ~10 cells cm^{-3} or less in the ~40 to 60°C warm sediments, while coal-bearing sediment layers had elevated cell concentrations. This ultra-deep microbial community is taxonomically distinct from typical shallower deep-biosphere communities found at this and other marine locations.

One Sentence Summary: Microbial life is barely detectable in ~1.5- to 2.5-km deeply buried sediment laden with biogenic methane but stimulated in coal-bearing horizons.

Main Text: Microbial life has been found in marine sediments buried up to 1,922 meters below the seafloor (mbsf) (*1*), with cell numbers decreasing logarithmically with increasing burial depth (*2*). However, the extent of the deep subseafloor biosphere and factors limiting or stimulating life at its lower boundaries remain largely unknown, partly due to the technological challenges associated with obtaining pristine scientific samples from great burial depths. Biodegradation of heavy oils points to the potential existence of microbial life in fossil fuel reservoirs of down to ~4 km depth [e.g., (*3, 4*)]; however, retrieval of microbiological samples from these hydrocarbon reservoirs was long beyond the technical capabilities of the scientific ocean drilling program.

We studied the microbial communities associated with deep subseafloor coalbeds that are widespread along the Western Pacific continental margins (*5*). During Integrated Ocean Drilling Program (IODP) Expedition 337 in 2012, we drilled and recovered sediments of marine and transitional environments down to 2,466 mbsf using the riser-drilling vessel *Chikyu* at Site C0020 in the northwestern Pacific margin (41°10.5983′N, 142°12.0328′E, 1,180 m water depth; fig. S1) (*6*). The site is located in the forearc basin offshore of the Shimokita Peninsula, Japan, where organic matter-rich sediments were deposited in coastal environments in the late Paleogene before the depositional environment turned marine in the course of subsidence (*5*). The series of ~1.9 to 2.5 km deeply buried lignite coalbeds are 0.3 to 7 m thick (*6*). Due to the relatively low geothermal temperature gradient of 24°C km^{-1} at this site (*6*), the deepest horizons are well within the temperature limits of microbial life (i.e., <60°C).

We detected intact microbial cells throughout the entire drilled sediment core down to the deepest sample at 2,458 mbsf. Cell concentrations decreased with depth, but in an unexpected fashion. In the "shallow subseafloor" above 365 mbsf, which was recovered during the *Chikyu* cruise CK06-06 from the top portion of the borehole in 2006 (*6, 7*), concentrations decreased steadily with depth from ~10^9 to ~10^7 cells cm^{-3} (*8*) and exceeded predictions based on the global regression line from previous surveys of cell concentrations at ocean margins (*2*) (Fig. 1A, table S1). By contrast, cellular concentrations in the "deep subseafloor" below ~1.5 km typically ranged from ~10^2 to 10^3 cells cm^{-3}, with local peaks in coal-bearing horizons (Fig. 1A).

These low cell concentrations required implementation of a rigorous contamination control in order to characterize the indigenous deep coalbed biosphere. To minimize and quantify the potential contamination of sediment samples introduced during drilling, we investigated whole round core sections by X-ray computed tomography, took microbiological samples from the center part of undisturbed core intervals, and monitored the intrusion of drill fluids into the core by perfluorocarbon tracer assays (*6*, *7*). As additional validation, we analyzed and sequenced the V1 to V3 region of 16S rRNA genes in all sediment samples (*7*) obtained by riser drilling in parallel to control samples consisting of drill mud and lab experimental blanks, to differentiate indigenous microbial communities from contaminant cells (fig. S2) (*7*). We applied a novel probabilistic approach incorporating taxon variability across samples to identify the likelihood that each taxon would be consistently sampled either exclusively or mutually from the control and sediment sample sets (*7*). In this way, we determined not only which taxa were found exclusively in the sediment sample sets ("most conservative") (figs. S2 to S4, table S1), but also cases of potentially indigenous taxa, which were inconsistently identified with low abundance in the contaminant samples, but consistently found with significant abundance in the sediment samples ("most ~~likely~~probable") (figs. S5 and S6, table S1). From this, we derived correction factors (table S1) to the raw cell concentrations to estimate the corresponding populations sizes. We used the results of both taxonomy-based sequence filtration and the probability-based relationship analysis for 16S rRNA gene sequences to estimate the "most conservative" and "most probable" indigenous cell concentrations, respectively (Fig. 1A, table S1) (*7*). These estimated population densities are drastically lower than predicted by the slope of the global regression line (*2*) and are even lower than previously reported values from one of the most oligotrophic subseafloor settings on Earth, the South Pacific Gyre (*9*).

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Despite the very low cell numbers, geochemical data indicate microbial activity even in the deepest sediment horizons sampled. Carbon isotopic compositions of methane (δ^{13}C-CH_4) and ratios of methane over ethane (C_1/C_2) (Fig. 1B and C), both continuously monitored in circulating mud gas during riser drilling (*6*), testify to microbial methanogenesis as the predominant source of methane (cf. *10*) throughout the entire drilled sedimentary sequence. Positive inflections of C_1/C_2 ratios between 1,700 and 2,000 mbsf suggest that biological methanogenesis is stimulated in coal-bearing horizons (Fig. 1C), where contamination-corrected cell concentrations reach ~10^2 to 10^4 cm^{-3} (Fig. 1A), as well as in the overlying 200 m of sediment. Hydrogen isotopic compositions of methane (δD-CH_4) range from -200‰ to -150‰ (Fig. 1B), consistent with its production by hydrogenotrophic CO_2 reduction (*10*). Local increases in δ^{13}C-CO_2 in coal-bearing horizons are further evidence for the CO_2 pool being isotopically fractionated by microbial methanogenesis (Fig. 1C). Moreover, *in situ* production of methane is supported by the abundance of $^{13}CH_3D$, a rare doubly substituted isotopologue of methane, in formation fluids sampled in two discrete coalbed horizons (Fig. 1B, table S2) (*6*,*7*). These analyses returned low $\Delta^{13}CH_3D$-based temperatures of 70^{+9}_{-9} and 70^{+24}_{-21} °C (table S2), thus arguing against substantial contributions of more deeply sourced, thermogenic methane, which would be expected to carry clumped-isotope temperatures >150°C (*11*). In addition, we detected coenzyme F_{430} in core samples, providing direct biomarker evidence for the activity of methanogenesis in ~2 km-deep coalbeds (Fig. 2A, table S3) (*12*, *13*). Coenzyme F_{430} is a key prosthetic group of methyl-coenzyme M reductase that catalyzes the last step of methanogenesis; its concentrations in deep sediments are ~two orders of magnitude lower than in shallow

sediments (table S3), indicating the presence of a small but persistent community of methanogens in deep coal-bearing layers.

In a continuous-flow bioreactor (cf. *14*) at near *in situ* temperature (40ºC), we successfully enriched methanogenic communities from ~2 km-deep coalbed samples that produced up to ~0.6 μM of methane after 35 days (Fig. 2B to 2G). Analysis of methyl-coenzyme M reductase genes (*mcrA*) indicated growth of hydrogenotrophic methanogens closely related to *Methanobacterium subterraneum* and *Methanobacterium formicicum* (fig. S7). These species have previously been detected in terrestrial coalbeds (*15*) and in shallower, methane hydrate-bearing sediments at Site C0020 (*14*). Using nano-scale secondary ion-mass spectrometry (*7, 16*), we detected the incorporation of ^{13}C-labeled bicarbonate into cellular biomass (Fig. 2E to G). Collectively, these microbiological and geochemical findings indicate that microbial communities are stimulated by the presence of coalbeds and that hydrogenotrophic methanogens act as terminal remineralizers.

Despite the geochemical and biomarker-based evidence for the activity of methanogenic archaea, we were not able to amplify archaeal 16S rRNA and *mcrA* genes by polymerase chain reaction (PCR) from the sediment core samples (*7*), except for two genes related to *Methanococcus maripaludis* and *Methanosarcina barkeri* (fig. S7). Archaeal 16S rRNA genes were neither quantifiable by digital PCR (*17*) nor stably amplifiable using multiple primer sets, indicating that this deep subseafloor microbial ecosystem harbors substantially lower proportions of Archaea than shallower sediments at this and other ocean margin sites (*18*). The difficulty to detect methanogenic archaea using multiple molecular assays is not unexpected given their generally low relative abundance of <1%, even in methane-laden subseafloor sediments (*19*).

Taxonomic distribution of the most conservative indigenous communities based on 16S rRNA gene sequences show that bacterial communities in “deep subseafloor” habitats (1,278 to 2,458 mbsf) differ profoundly from “shallow subseafloor” communities (0-364 mbsf) (Fig. 3, table S4, figs. S3 and S4). For example, lower proportions of sequence reads affiliated with the phyla Chloroflexi or ‘Atribacteria’ [candidate division JS1 (*20, 21*)], both globally abundant groups in subseafloor sediments at ocean margins (*22, 23*), were detected in the deep layers (Fig. 3A, table S4, fig. S3). The sequence assemblage in deep layers is mostly represented by heterotrophic bacteria with close relatives commonly found in forest soils or organic-rich freshwater environments (Fig. 3A, table S4, fig. S4); however, the strong difference in composition and distribution between the deep assemblage and subsurface soil ecosystems (e.g., ref. *24*) suggests that the conditions in the deep habitats have selected for this residual population (e.g., Gemmatimonadetes, Synergistetes: fig. S8) (*7*). Beta-diversity analyses indicate the existence of bacterial communities that statistically differ from those found in shallow and deep sedimentary habitats. Clustering and Bray-Curtis dissimilarity analyses (Fig. 3B) as well as multidimensional scaling analysis (fig. S9) based on family/genus-level classification show that there are distinguishable clusters aligned with distinct depositional settings: deep-sea diatomaceous sediment, lacustrine or shallow marine sediment, and wetland or peat-derived coaly sediment (Fig. 3B).

Our combined microbial and geochemical dataset provides an opportunity to examine the factors that limit microbial life below ~1.5 kmbsf. The concentration of dissolved hydrogen (H_2), a key intermediate in the anaerobic degradation of organic matter, is an important gauge of the bioenergetic status of anaerobic microbial ecosystems (*25, 26*). At Site C0020, the uniformly high H_2 concentrations of ~1 to ~500 μM in sediments below 1.5 km (fig. S10, table S5) result in

Gibbs free energy yields of hydrogenotrophic methanogenesis that are much more negative than those previously documented from energy-rich surface environments (fig. S11, tables S6 and S7; ref. *25*). These high H_2 concentrations suggest very low H_2 turnover rates, and might be the direct consequence of low population densities of microorganisms with low viabilities and consequently low cell-specific energy turnover. Under these circumstances the coupling between substrate production and substrate uptake may be severely delayed resulting in long residence times and accumulation of H_2 to high concentrations in sediment porewater. Despite the high H_2 concentrations, a range of organotrophic reactions involving the breakdown of cellular building blocks and other intermediates are likely to be thermodynamically favorable, and explain the presence of alive and active microbial populations (fig. S11).

In addition to energy from organotrophic reactions, an important factor controlling the viability and size of microbial communities buried more deeply than 1.5 km could be the increase in energy expended on the repair of biomolecules. Abiotic amino acid racemization and DNA depurination are biomolecule-damaging reactions, the rates of which increase exponentially with temperature (*27*) (fig. S12). Substantial increases in both modeled amino acid racemization and DNA depurination rates with depth at Site C0020 coincide with a dramatic drop in cell numbers. The increased energetic cost of biomolecule repair could result in a higher cell-specific energy demand in the ultra-deep habitats at Site C0020 and explain why microbial abundance is only a small fraction of the size predicted by the global regression line.

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Supplementary Materials:

Materials and Methods

Figures S1-S12

Tables S1-S7

References (*28*-*79*)

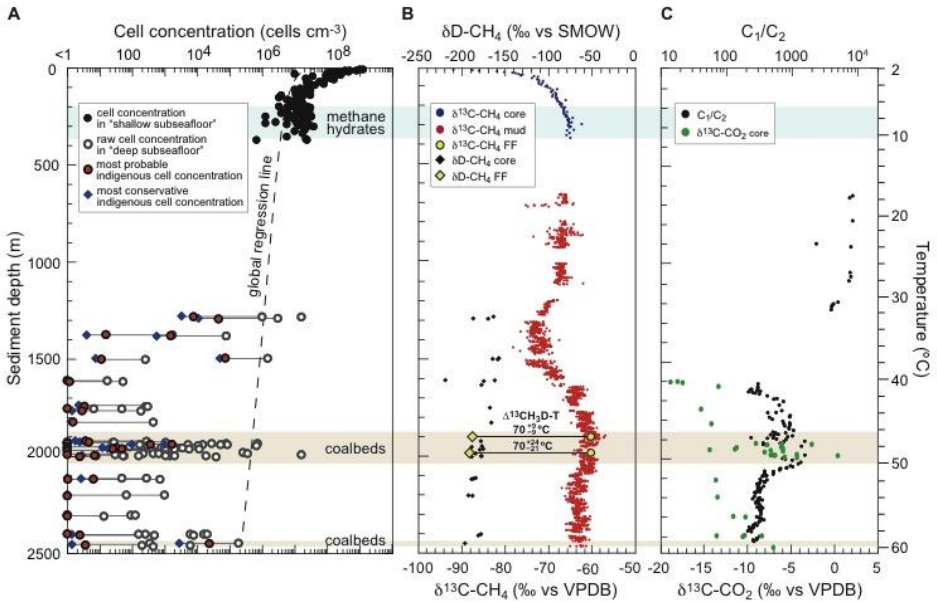

Fig. 1. **Depth profiles of microbial cell counts and geochemical data at Site C0020. (A)** microbial cell concentrations, **(B)** $\delta^{13}C$ and δD of methane, and **(C)** C_1/C_2 ratios and $\delta^{13}C$ of CO_2. (A) For cell concentrations in "deep subseafloor", raw data of fluorescence image-based cell counts (*8*), the most conservative indigenous cell concentrations estimated based on the taxonomic classification (*7*), and the most probable indigenous cell concentrations based on the probability-relationship set analysis (*7*) are shown (cf. table S1). Based on raw cell concentrations and the proportion of 16S rRNA gene sequence reads most conservatively identified as members of the indigenous population (*7*; table S1), the minimal estimate of *in situ* microbial cell numbers per sample was estimated as follows: n' = a/b*n, where n'= indigenous cell count, n = raw cell count, a = number of sequences remaining after removal of potential contaminant sequence reads, and b = total number of reads sequenced. The correction factor a/b is the proportion of sequences estimated to be indigenous. The minimal quantification limit for raw cell counts was 1.43×10^2 cells cm^{-3}; i.e., the upper 95% confidence interval of negative background. All $\delta^{13}C$ and δD in (B) and (C) are in ‰ versus Vienna Pee Dee Belemnite (VPDB) and Standard Mean Ocean Water (SMOW), respectively. The $\Delta^{13}CH_3D$-T values designate the apparent equilibrium temperatures derived from measurements of methane's clumped isotopologue $^{13}CH_3D$ (table S2) in discrete formation fluid (FF) samples (*6*, *7*). Temperature is based on the temperature gradient of 24.0ºC km^{-1} determined by downhole logging (*6*).

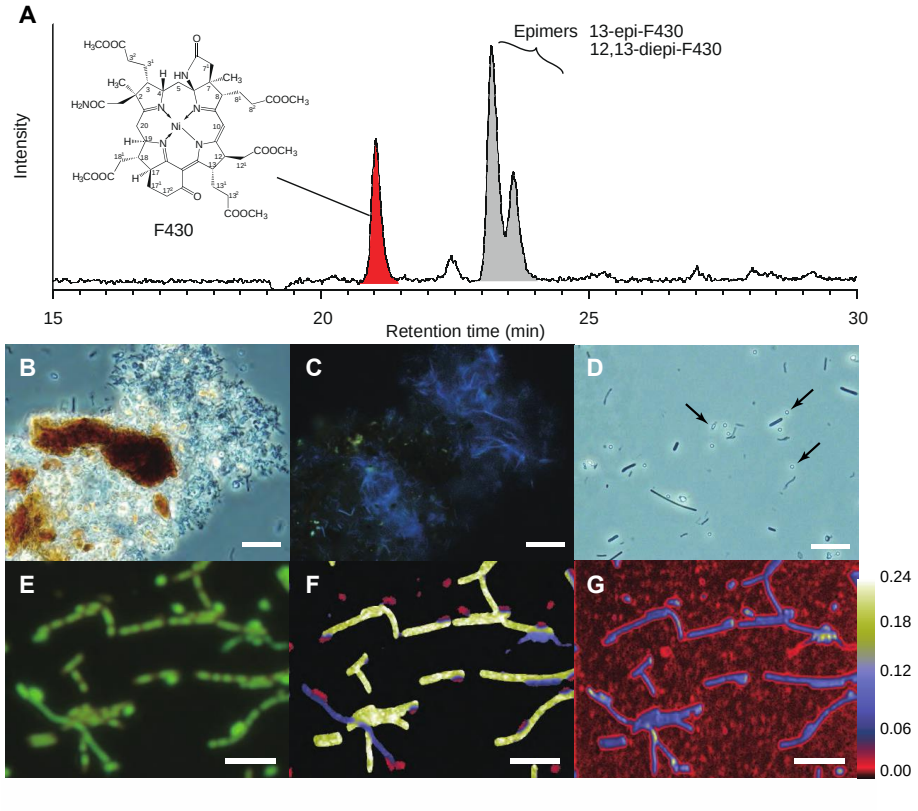

Fig. 2. Geochemical and microbiological indications for methanogenic microbial communities in ~2-km-deep subseafloor coalbeds. (**A**) A representative chromatogram of the diagnostic methanogen biomarker intact F_{430} and its epimers from a coal sample (Core 18R-2, 1,946 mbsf; table S3). (**B-D**) Photomicrographs of an enrichment culture from ~2-km-deep coalbed samples using a continuous-flow bioreactor (*7*). (B) Phase-contrast micrograph of microbial cells attached to mineral particles. (C) Fluorescent micrograph of the same field of (B) shows growth of methanogens that produce auto-fluorescence derived from coenzyme F_{420}. (D) Phase-contrast micrograph shows spherical spore-like particles indicated by arrows. (**E-G**) Nano-scale secondary ion-mass spectrometry (NanoSIMS) analysis of cells in the reactor enrichment culture incubated with ^{13}C-labeled bicarbonate (*7*). (E) Fluorescent micrograph of SYBR Green I-stained cells. (F and G) NanoSIMS ion image of (F) $^{13}C/^{12}C$ and (G) ^{12}C, color gradient indicates relative ^{13}C abundance expressed as $^{13}C/^{12}C$. Bar length is 10 μm.

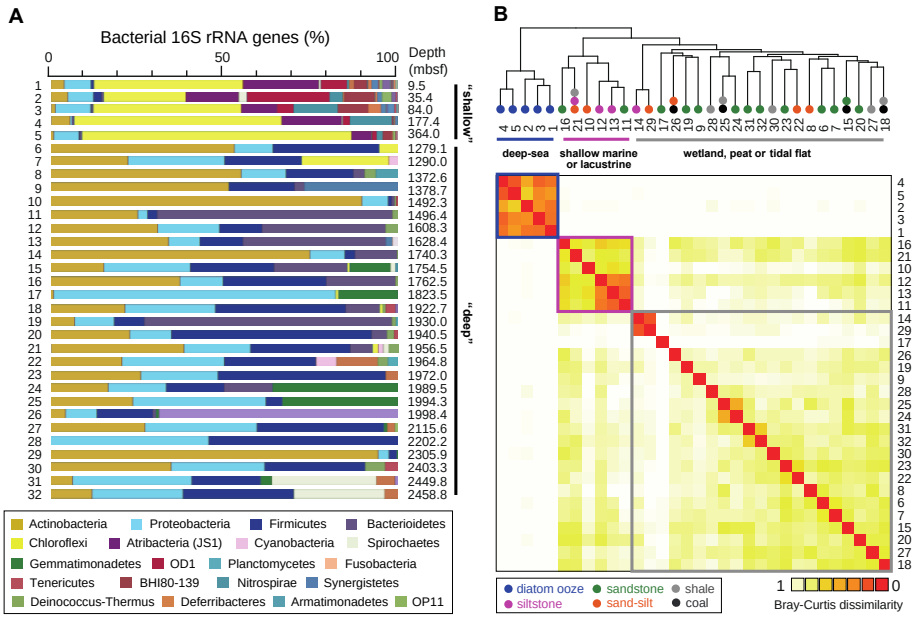

Fig. 3. Taxonomic distribution of the most conservative indigenous bacterial communities in sediments at Site C0020. (**A**) Phylum/class-level taxonomic composition of bacterial 16S rRNA gene-tagged sequences (i.e., V1-V3 region) in "shallow subseafloor" (no. 1 to 5, 9.5-364 mbsf from the *Chikyu* cruise CK06-06) and "deep subseafloor" (no. 6 to 32, 1279.1-2458.8 mbsf from the IODP Expedition 333) sediment samples (*7*). (**B**) Cluster and Bray-Curtis dissimilarity analysis of bacterial community structure based on the family/genus-level classification of the same sequence assemblages used in (A). Color dots and lines represent lithological characteristics and depositional environment of each sample horizon, respectively (*7*).